BMC Infectious Diseases

RESEARCH

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Investigating the relationship between lymphocyte cells apoptosis and DNA damage and oxidative stress and therapeutic and clinical outcomes of COVID-19 elderly patients

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Abstract

Background While COVID-19 has been controlled and deaths have decreased, the long-term consequences of COVID-19 remain a challenge we face today. This study was conducted to determine the relationship between the apoptosis of lymphocyte cells with DNA damage and oxidative stress and the therapeutic and clinical outcomes of elderly patients with COVID-19.

Methods This study was conducted from April 2020 to May 2021 (the period of severe attacks of the epidemic peak of COVID-19) and September 2022 (the post-COVID-19 period). The study groups included elderly patients with COVID-19 hospitalized in the ICU and normal wards of the hospital as well as elderly patients with influenza. A polymerase chain reaction was used to check the validity of the studied diseases. The Annexin V/Propidium Iodide method was used to evaluate the level of apoptosis. Genotoxic effects and DNA damage were assessed by the comet assay method. Total antioxidant status (TAS), total oxidant status (TOS), and myeloperoxidase activity (MPO) were measured by photometric methods.

Results The highest level of apoptosis in peripheral blood lymphocytes and the highest level of DNA damage were observed at both times in the intubated-ICU and non-intubated-ICU groups. In all groups, there was a significant increase in peripheral blood lymphocyte apoptosis levels and DNA damage levels compared to the healthy control group ($p < 0.01$). The level of apoptosis and DNA damage decreased significantly in the post-COVID-19 period ($p < 0.01$). In the investigation of oxidative stress biomarkers, the oxidative stress index, including TOS and MPO levels, increased in patients ($p < 0.01$), and the TAS level decreased ($p < 0.01$).

Conclusion It shows that the apoptosis of lymphocyte cells, DNA damage, and oxidative stress can be effective in prognostic decisions and is a suitable predictor for diagnosing the condition of patients with viral infections such as COVID-19 and influenza.

Keywords Apoptosis, COVID-19, Lymphocyte, Lymphopenia, Flow cytometry, Influenza virus, Pandemic, DNA damage, TAS, TOS, MPO

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Background

The devastating event of acute respiratory syndrome COVID-19, until today, has killed thousands of people worldwide. It can be said that the reasons that caused this global pandemic to become a big problem include its unexpected spread in society, the unknown nature of this virus, and the unknown ways to confront it. COVID-19 was first identified in Wuhan, China, in December 2019 [1]. Continuous research has shown that the sequence homology of the coronavirus is similar to that of SARS-CoV-2 [2]. In 2002–2003, severe acute respiratory syndrome (SARS), in 2012, Middle East respiratory syndrome (MERS), and in December 2019, novel human disease (COVID-19) coronaviruses (RNA viruses) caused severe respiratory pandemics. The World Health Organization (WHO) introduced SARS-CoV-2 as the agent of the novel coronavirus infectious disease (COVID-2019) on January 12, 2020 [3]. Dry cough, fatigue, fever, shortness of breath, muscle pain, diarrhea, and pneumonia are clinical symptoms of COVID-19. It manifests as a multisystem, multifactorial, and multiorgan disease, and pathogenic agents can affect multiple organs. COVID-19 affects all age groups, but in elderly patients with underlying comorbidities, and then in all age groups of patients with underlying co-morbidities, the probability of experiencing a more severe disease is higher [4, 5]. By international definitions, COVID-19 falls within the scope of septic syndromes (organ failure due to an abnormal host`s response to infection), which is a major factor in mortality due to suppression. Also, sepsis is in the same range. Some patients with COVID-19 show severe lymphopenia and an inflammatory response, similar to sepsis [6, 7]. Patients with COVID-19 may have acute respiratory distress syndrome (ARDS) with additional clinical symptoms [6]. Gradually, the severity of COVID-19 progresses, and ARDS occurs in some severe cases, so a flow cytometry examination may provide important information about the inflammation or suppression of the patient's immune system and the progress of the disease [8]. Recent findings in COVID-19 patients show immunosuppression, which includes a continuous reduction of mHLA-DR expression, a reduction of monocyte subsets (CD14lowCD16+), the release of inflammatory cytokines, lymphopenia, a significant reduction in peripheral blood lymphocyte count in most patients, cytokine release syndrome (also seen in the severe influenza pandemic in 2009), altered IFN-α production, and a low plasmacytoid dendritic cell count. This scenario is important for patients with COVID-19 and suggests that the weakness of the host`s immune system is effective against the development of COVID-19 [3, 6, 9]. Generally, irregular immune responses, disease severity, and increased mortality are related to the above-mentioned features in COVID-19 patients [10, 11]. These abnormalities are observed more in elderly people, who are significantly special victims of COVID-19 [6]. The coexistence of incurable diseases, especially in the elderly, medical services and the quality of service delivery, weakness of the immune system, and old age may be various factors that increase the severity of the disease. Age above 60 years is known to be an important factor in increasing the severity of illness and mortality rate of patients with COVID-19 [12–18]. Until today, there are no vaccines or antibiotics that work against COVID-19, and there is not even an established immune response to be useful against this completely new and unknown virus. Therefore, observations show that elderly patients with immunosuppression stay longer in the ICU departments of hospitals and experience a worse condition of this disease [19]. These issues may explain why patients with COVID-19 in the ICU remain much less immune-modulated than those with other bacterial ARDs treated with antibiotics. Suitable approaches that can be given more special attention include the inflammatory process in addition to immune stimulation in COVID-19 and possibly the spread of the pulmonary virus instead of the systemic cytokine storm [20–22]. Systematic self-destruction occurs in single cells of multicellular organisms in response to various stimuli, such as viral infection, leading to apoptosis, a genetically controlled, preprogrammed event. In studies, several viruses with different viral gene products induce apoptosis in vitro and in vivo [23]. Influenza virus is a powerful pathogen that can lead to lymphocyte apoptosis. One of the important health problems that have existed worldwide since the past and until today is respiratory infections, one of the main causes of which is influenza A virus (IAV). The risks of respiratory infections mostly threaten the elderly. Although influenza affects children very quickly, the elderly have the highest death rate due to this viral infection. Types of influenza viruses, including relatively mild H1N1pdm2009 viruses and pandemic IAV infections, can lead to death, especially in the elderly and immunocompromised people. Advanced respiratory viral infection is directly related to lymphopenia. Lymphopenia is caused by several mechanisms, especially a viral infection that directly infects cells and produces a toxic effect. Influenza readily infects PBMC cells in humans [24]. Comparing the pattern of apoptosis of lymphocytes, levels of DNA damage, and levels of oxidative stress in different groups in mild and severe conditions of RNA virus infection allows the use of these biomarkers as prognostics to prevent further risk before it occurs.

Methods

Controls and patients

After obtaining signatures and obtaining informed consent from the patients, blood samples were collected from patients admitted to Imam Hossein Shahrood Hospital affiliated with Shahrood University of Medical Sciences at two different times and transferred to the laboratory. The first time was chosen during the epidemic peak of COVID-19 (from April 2020 to May 2021), and the second time was chosen in the post-COVID-19 period (September 2022). This article is in accordance with the Declaration of Helsinki, and the code of ethics was approved by the ethics committee of Damghan Azad University with the number (IR.IAU. DAMGHAN. REC.1400.005). The first time, 124 patients with COVID-19 (62 men and 62 women) and 40 patients with influenza were hospitalized, and the second time, 80 patients with COVID-19 (40 men and 40 women) and 20 patients with influenza were hospitalized in different departments of the hospital. The healthy control group had similar demographic characteristics, especially an age over 60 years and no underlying disease. Elderly COVID-19 patients studied for the first time who were admitted to the hospital and were randomly selected included 40 people intubated in the intensive care unit, 40 non-intubated people in the ICU, 40 people in the normal ward of the hospital, 40 people infected with influenza, and 40 healthy people (20 men and 20 women). Elderly COVID-19 patients studied in the second period who were admitted to the hospital and were randomly selected included 20 intubated people in the ICU department, 20 non-intubated people in the ICU special care department, and 20 people hospitalized in the normal hospital department. Twenty patients with COVID-19 who were vaccinated against COVID-19 in the last 6 months and were hospitalized in the normal department of the hospital, 20 people with influenza, and 20 healthy elderly people (10 men and 10 women) were also included. Their entry and exit conditions are listed below. The proof of people's infection with the COVID-19 virus was done by observing clinical symptoms and then taking laboratory samples and analyzing them based on the protocols of the Ministry of Health, Treatment, and Medical Education. The criteria for entering the study are as follows: obtaining written consent to participate in the research, being older than 60 years old [25–27], not suffering from infectious diseases (at least in the last 30 days from the time of entering the study) in a group of healthy people. In this study, mild and severe clinical grades were classified based on the new coronavirus protocols published by the Ministry of Health. The clinical and demographic data of the collected confirmed patients with COVID-19 are aggregated in Table 1. The clinical laboratory of Imam Hossein Shahrood Hospital is the place to collect all medical laboratory data. People who had all the symptoms related to the influenza disease and whose RT-PCR test did not detect SARS-CoV-2 were identified as patients with influenza and were examined and tested. The nucleic acid of the SARS-CoV-2 virus was discovered in all patients with COVID-19 with the help of an RT-PCR test. The detailed protocol has been described previously [27, 28]. Oropharyngeal and nasopharyngeal (NP/OP) swabs belonging to patients were collected, and they were used to diagnose acute respiratory viral infections,

Table 1 Demographics of patients infected with COVID-19 and influenza in 2020 (pandemic peak of COVID-19) and in 2022 (post-COVID-19)

Number (%)							
Epidemic peak of COVID-19	Characteristics	All COVID-19 patients in 2020 ($n=124$)	Non-ICU ($n=44$)	Non-intubated -ICU ($n=40$)	Intubated-ICU ($n=40$)	Influenza ($n=40$)	
	Age, median (IQR), years Sex Male Female	65 (60–93) 62 (50%) 62 (50%)	60 (60–63) 22 (50%) 22 (50%)	66 (64–71) 20(50%) 20(50%)	68 (66–93) 20 (50%) 20 (50%)	61 (60–72) 20 (50%) 20 (50%)	
Post-COVID-19	Characteristics	All COVID-19 patients in 2022 ($n=80$)	Non-ICU ($n=20$)	Non-intubated -ICU ($n=20$)	Intubate-ICU ($n=20$)	Vaccinated COVID-19 patients ($n=20$)	Influenza ($n=20$)
	Age, median (IQR), years Sex Male Female	70 (52–103) 40 (50%) 40 (50%)	65 (64–67) 10 (50%) 10 (50%)	72 (70–88) 10 (50%) 10 (50%)	73 (75–103) 10 (50%) 10 (50%)	59 (51–99) 10 (50%) 10 (50%)	62 (52–70) 10 (50%) 10 (50%)

including COVID-19 and influenza. Chemagic 360 technology (PerkinElmer Inc.) was used to extract RNA from clinical samples. A viasure SARS-CoV-2 RT-PCR detection kit (Certest Biotec SL, Spain) was used to identify N1 and N2 genes belonging to the virus related to COVID-19, and a viasure respiratory viral panel I RT-PCR detection kit (Flu A, Flu B, and RSV) (Certest Biotec SL, Spain) was also used [28].

Blood sample collection

Six milliliters of blood samples from the patients were extracted and put into sterile blood tubes with EDTA. A volume of 200–400 µl was taken out of this blood and placed in 1.5-ml centrifuge tubes. The remaining EDTA blood was centrifuged at 3000×g after 10 min. Its plasma was centrifuged and then kept at − 80 °C for examination.

Flow cytometric analysis

Two-color flow cytometry was analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific). Peripheral blood mononuclear cells (PBMCs) were washed twice in 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES buffer (Gibco)). Three × 105 cells were added in 50 µl of rebinding buffer; then, 1 µl of Annexin V-FITC was added and incubated at room temperature in the dark for 15 min. PI (0.25 µg/ml) was added and analyzed, and the analysis was done. A total of 40,000 events were acquired at low speed. The doubling of the population is thought to be due to late apoptosis. PI-positive and Annexin V populations were considered early apoptosis and necrosis cells, respectively. With the FlowJo software (treestar), the results of each sample were analyzed. A combination of fluorescein, Annexin V-FITC, and PI was used to stain and detect dead cells, late apoptosis/necrotic cells, early apoptosis cells, and non-apoptosis cells.

Alkaline single-cell gel electrophoresis

The comet assay method of alkaline single-cell gel electrophoresis was utilized to evaluate the damage to leukocyte DNA [29]. This was accomplished by mixing 0.7% low-melting-temperature agarose with 6 µl of frozen whole blood and then embedding the mixture on slides coated with 1% normal melting-temperature agarose gel. After coating, the coverslip was placed in a cool environment to solidify. After the gel had been set, the coverslips were removed from the slide, and the cells were lysed in a lysis solution for at least 4 h. After that, they were electrophoresed for 20 min at 300 mA in an alkaline buffer with a pH of 13. Fluorescence microscopy was used to examine the cells labeled with ethidium bromide (5 mg/ml) after electrophoresis (emission DB: 20 nm, excitation DB: 546 nm). The DNA damage marker, DNA tail density (tail%), was investigated. Comet analyses were performed using the comet assay analysis program IV (Perceptive Instruments, Suffolk, UK), with an average of 50 cells counted.

Measurement of total oxidant status (TOS) and total antioxidant status (TAS)

Erel's approach [30] was used to examine the plasma samples' overall amounts of antioxidants. The test's foundation is the way antioxidants in the sample break down the blue-green hue that the ABTS (2,2′-azinobis-3-ethylebenzothiazo-line-6-sulfonate) radical forms. To create the ABTS + radical, ABTS is incubated with a peroxidase that contains myoglobin (HX-Fe +) and H_2O_2. The resulting blue-green Ferrell myoglobin combines with ABTS to create the ABTS + radical. Using a varioskan multimode reader (Thermo Scientific, USA) at 600 nm, this generated color is prevented based on the ratio of antioxidants in a sample. TAS was computed using ascorbic acid as a benchmark. Erel's approach [31] was employed to ascertain the levels of total oxidants in plasma. The oxidation of ferric ions to ferrous ions in the presence of various oxidant species is what determines the results of the total oxidant test. The iron-a-dianisidine compound is oxidized by the oxidants. The resulting colorful complex of xylenol orange-ferric ion is formed by the generated ferric ion. The intensity of color in a sample is dependent on the amount of oxidant present. A varioskan multimode reader was used to measure this color change at a wavelength of 530 nm. H_2O_2 was used as a standard for computing TOS. The formula for calculating the oxidative stress index (OSI) was TOS/TAS.

Measurement of myeloperoxidase (MPO) enzyme activity

The modified o-dianisidin-H_2O_2 technique was used to measure the plasma MPO enzyme activity of the samples in 96-well plates. Twenty microliters of plasma samples were mixed with 50 mmol/l of potassium phosphate buffer (pH 6.0) and 0.53 mmol/l of o-dianisidine (dihydrochloride). Then, at room temperature, it was incubated for ten minutes. Following the incubation period, the absorbance change (ε = 10,062/M/cm) was determined. For 10 min, results are expressed in U/L.

Analytical statistics

The software GraphPad Prism version 8.0 was utilized to create the study's graphs and statistical analysis. The continuous variables with interquartile ranges (IQRs) were described using the mean ± standard deviation or median. The categorization factors were explained using frequency or percentage. Quantitative data was shown to be normal by the Kolmogorov–Smirnov test. In order to compare more than two independent parameters, the

Kruskal–Wallis test was employed. The chi-square test was used to evaluate the categorical data. Variables were compared using Fisher's exact test. $p < 0.01$ was considered significant. Within different groups, the means of quantitative variables were compared with an analysis of variance (ANOVA) test.

Results

Clinical and laboratory findings of patients with COVID-19 and patients with influenza

In this study, 264 patients with COVID-19 and patients with influenza in 2020 and 2022 (132 men and 132 women) with a mean age of more than 60 years were studied (Table 1).

Clinical signs were not significantly different in mild and severe cases (Table 2). The highest symptom in all COVID-19 patients is related to chest pain (88.7%), and the lowest symptom is related to dry cough (4.03%). The highest symptom in the non-ICU group is chest pain (84.0%), and the lowest symptom is dry cough (4.54%). The highest symptom in the non-intubated-ICU group is related to chest pain (85%), and the lowest symptom is related to anorexia (2.5%) and dry cough (2.5%). The highest symptom in the intubate-ICU group is related to chest pain (97.5%), and the lowest symptom is related to anorexia (0%). The highest symptom in the influenza group is related to fatigue (85%), and the lowest symptom is related to diarrhea (5%).

About 95.1% (118 people) of the hospitalized COVID-19 patients in 2020 were rescued, and 4.83% of them died. About 54.8% (68 people) of the participants were male. The average age of people who died due to COVID-19 was 75.28 years, which was higher than the average age of people who survived (60.46 years), which was statistically significant ($P < 0.01$). Furthermore, a statistically significant proportion of patients who passed away had hypertension and cardiovascular illness ($p < 0.01$). The demographics of COVID-19 deaths by patient characteristics in 2020 are shown in Table 3. Most of the COVID-19 patients who had an underlying disease had hypertension (23.3%), and 50% of the 6 COVID-19 patients who died due to this disease had hypertension. In the results, 20.1% of the COVID-19 patients had a digestive system disorder, and 33.3% of the deceased had a digestive system disorder at the same time. Among the patients with COVID-19, 19.3% had inflammation, and 16.6% of the patients who died also had the same underlying disease. Also, 16.1% were suffering from cardiovascular disease, and 50% of the deceased patients had the same underlying disease, which indicates the dangerousness of this underlying disease. The lowest percentage of underlying disease in COVID-19 patients belongs to diabetes (15.3%), and 16.6% of the deceased had the same underlying disease. Also, 34.6% of COVID-19 patients were without comorbidity. In the results, 15.3% of COVID-19 patients had one underlying disease, and 16.6% of the deceased had the same condition. Fifty percent of COVID-19 patients had two or more underlying diseases, which indicates the dangerousness of this condition, and 66.6% of the patients who died had the same condition. The most symptoms in COVID-19 patients are related to cough (63.7%), and the least symptoms in COVID-19 patients

Table 2 Symptoms of patients infected with COVID-19 in 2020 (pandemic peak of COVID-19)

Number (%)						
Characteristics	**All COVID-19 patients in 2020 (*n* = 124)**	**non-ICU (*n* = 44)**	**non-intubated-ICU (*n* = 40)**	**Intubated-ICU (*n* = 40)**	**Influenza (*n* = 40)**	***p* value***
Signs/symptoms						
Dry cough	5 (4.03%)	2 (4.54%)	1 (2.5%)	2 (5%)	13(32.5%)	0.001
Chest pain	110 (88.7%)	37 (84.0%)	34 (85%)	39 (97.5%)	28 (70%)	0.001
Fatigue	82 (66.1%)	31 (70.4%)	29 (72.5%)	22 (55%)	34 (85%)	0.001
Fever	19 (15.3%)	7 (15.9%)	8 (20%)	4 (10%)	16 (40%)	0.001
Sputum production	25 (20.1%)	5 (11.3%)	12 (30%)	8 (20%)	3 (7.5%)	0.001
Diarrhea	9 (7.25%)	4 (9.0%)	2 (5%)	3 (7.5%)	2 (5%)	0.001
Vomiting	73 (58.8%)	29 (65.9%)	26 (65%)	18 (45%)	8 (20%)	0.001
Dyspnea	21 (16.9%)	9 (20.4%)	4 (10%)	8 (20%)	3 (7.5%)	0.001
Nausea	35 (28.2%)	17 (38.6%)	12 (30%)	6 (15%)	5 (12.5%)	0.001
Headache	71 (57.2%)	33 (75%)	15 (37.5%)	23 (57.5%)	21(52.5%)	0.001
Anorexia	23 (18.5%)	11 (25%)	1 (2.5%)	0 (0%)	8 (20%)	0.001

Abbreviations: COVID-19 coronavirus infectious disease, *IQR* interquartile range

* *p* values indicate differences between mild and severe patients. $p < 0.01$ was considered significant

Table 3 Demographics COVID-19 deaths by patient characteristics of COVID-19 in 2020

Characteristics	Number (%) all patients (*n* = 124)	Alive (*n* = 118)	Deaths (*n* = 6)	*p* value*
Age, median (IQR), years	65 (60–93)	60 (58–96)	75 (67–89)	0.001
Sex				
Male	68 (54.8%)	64 (54.2%)	4 (66.6%)	0.001
Female	56 (45.1%)	54 (45.7%)	2 (33.3%)	0.001
Groups				
All patients	124 (100%)	118 (95.1%)	6 (4.83%)	0.002
Non-ICU	44 (35.4%)	44 (35.4%)	0 (0%)	0.003
Non-intubated-ICU	40 (32.2%)	39 (97.5%)	1 (2.5%)	0.001
Intubated-ICU	40 (32.2%)	35 (87.5%)	5 (12.5%)	0.001
Comorbidity				
Underlying comorbidities	20 (16.1%)	17 (14.4%)	3 (50%)	0.003
Cardiovascular disease	29 (23.3%)	26 (22.0%)	3 (50%)	0.004
Hypertension disorder	25 (20.1%)	23 (19.4%)	2 (33.3%)	0.001
Digestive system	16 (12.9%)	15 (12.7%)	1 (16.6%)	0.005
Chronic intestinal inflammation	24 (19.3%)	23 (19.4%)	1 (16.6%)	0.003
Diabetes	19 (15.3%)	18 (15.2%)	1 (16.6%)	0.12
Without comorbidity	43 (34.6%)	42 (35.5%)	1 (16.6%)	0.003
With one underlying disease	19 (15.3%)	18 (15.2%)	1 (16.6%)	0.17
With two or more underlying diseases	62 (50%)	58 (49.1%)	4 (66.6%)	0.002
Symptoms				
Fever	65 (52.4%)	61 (51.6%)	4 (66.6%)	0.001
Chills	20 (16.1%)	19 (16.1%)	1 (16.6%)	0.15
Cough	79 (63.7%)	78 (66.1%)	3 (50%)	0.004
Dyspnea	73 (58.8%)	68 (57.6%)	5 (83.3%)	0.003
Anorexia	28 (22.5%)	22 (18.6%)	6 (100%)	0.001

The data are shown as median (interquartile range) or number/total (%). **p* Values indicate differences between alive and deaths patients. $p < 0.01$ was considered significant

are related to chills (16.1%). The most symptoms in deceased COVID-19 patients are related to anorexia (100%), and the lowest symptoms in deceased COVID-19 patients are related to chills (16.6%).

Comet assay

As an indicator of DNA damage, the tail DNA percent parameter was examined. The DNA damage is given as a percentage of the density of the tail. DNA damage in all COVID-19-affected groups showed a significant increase compared to the control group ($p < 0.01$) in the analysis of the tail DNA percent parameter. Additionally, in each COVID-19-affected group, a significant increase was observed during the epidemic peak of COVID-19 compared to the post-COVID-19 period ($p < 0.01$). The non-ICU, intubated-ICU, and non-intubated-ICU groups show the only statistically significant increase in damage at both times ($p < 0.01$). Figure 1 displays the average damage in each group.

Assessment of apoptosis of lymphocytes in severe and mild clinical stages of elderly patients with COVID-19

The percentage of apoptosis of peripheral blood lymphocytes in the severe clinical stage of COVID-19 patients had increased significantly compared to patients in the mild stage of the disease ($p < 0.01$). According to the results of this study, the percentage of apoptosis cells increases with the progression of the disease. Figure 2 shows the fluorescence intensity of Annexin V-FITC/PI in elderly COVID-19 patients in 2020 in different departments of the hospital. The highest apoptosis of peripheral blood lymphocytes is observed in the patients hospitalized in the ICU department, which is significantly different from the healthy control group ($p < 0.01$). Information

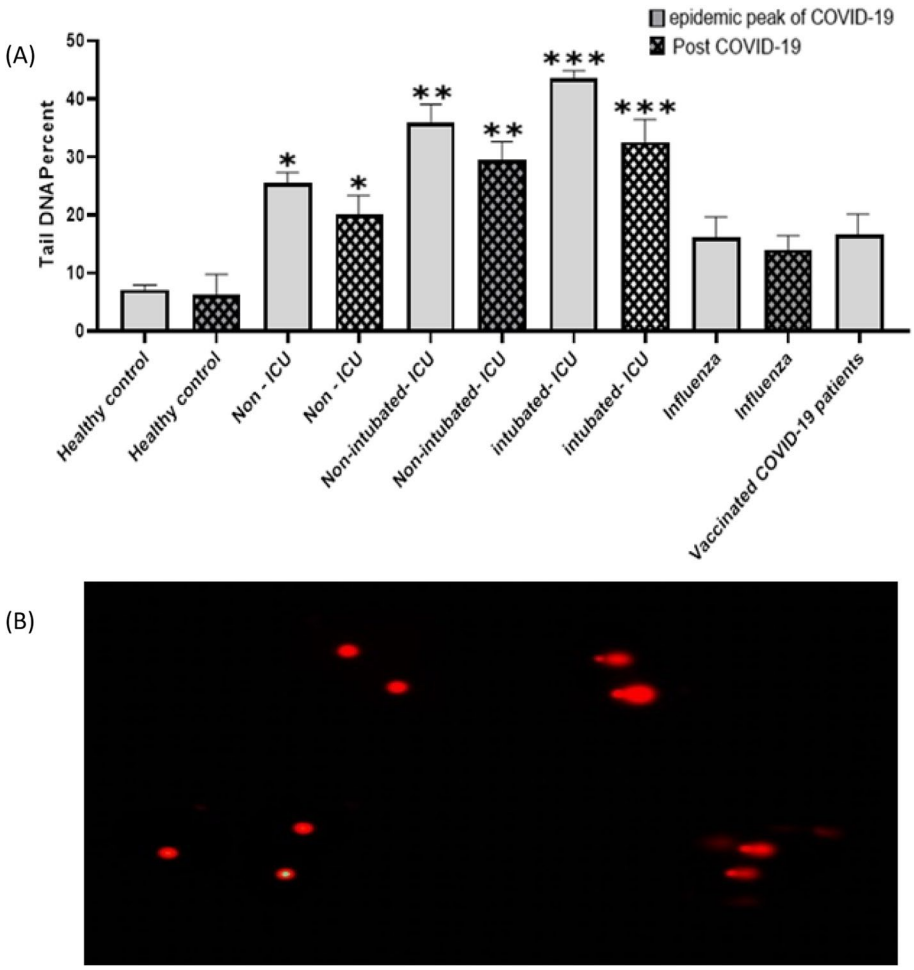

Fig. 1 **A** Average of the comet assay indices in the COVID-19 and influenza groups (**P*-value < 0.01). **B** Schematic figure of DNA damage obtained by the comet assay technique

is presented in Annexin V-FITC fluorescence intensity units and the number of cells counted. Row Q1 indicates necrotic cells, Q2 was considered for late apoptosis, Q3 for early apoptosis, and row Q4 in each analysis identifies non-apoptotic cells as Annexin V-FITC negative populations.

The average graphs in Fig. 3 show the percentage of apoptosis cells in the control group and elderly patients with COVID-19 in severe and mild clinical stages. By examining lymphocyte apoptosis in peripheral blood in all 3 groups of COVID-19 patients at both times, vaccinated COVID-19 patients in 2022, influenza groups, and comparison with the control group, in the control group, the percentage of lymphocyte apoptosis was significantly lower than in patients elderly with COVID-19 ($p < 0.01$). The percentage of lymphocyte apoptosis in patients with COVID-19 in 2020 compared to patients with COVID-19 in 2022, which is called the post-COVID-19 era, had significantly increased ($p < 0.01$). The apoptosis of peripheral blood lymphocytes has been significantly reduced in vaccinated COVID-19 patients. However, the injury in this group is not significantly different from patients with influenza ($p > 0.01$).

Oxidative stress indicators

In comparison to the healthy control group, Fig. 4 demonstrates that the COVID-19 patients had significantly greater levels of oxidative damage in the TOS and MPO, all of which demonstrated oxidative stress ($p < 0.01$). In terms of TOS, the influenza, non-intubated-ICU, and intubated-ICU groups significantly increased during the epidemic peak of COVID-19 compared to the post-COVID-19 period ($p < 0.01$). MPO significantly increased in all groups during the epidemic peak of COVID-19 outbreak compared to the post-COVID-19 period ($p < 0.01$). Antioxidant capacity (TAS) levels were statistically significantly reduced in COVID-19 patients. Specifically, compared to post-COVID-19,

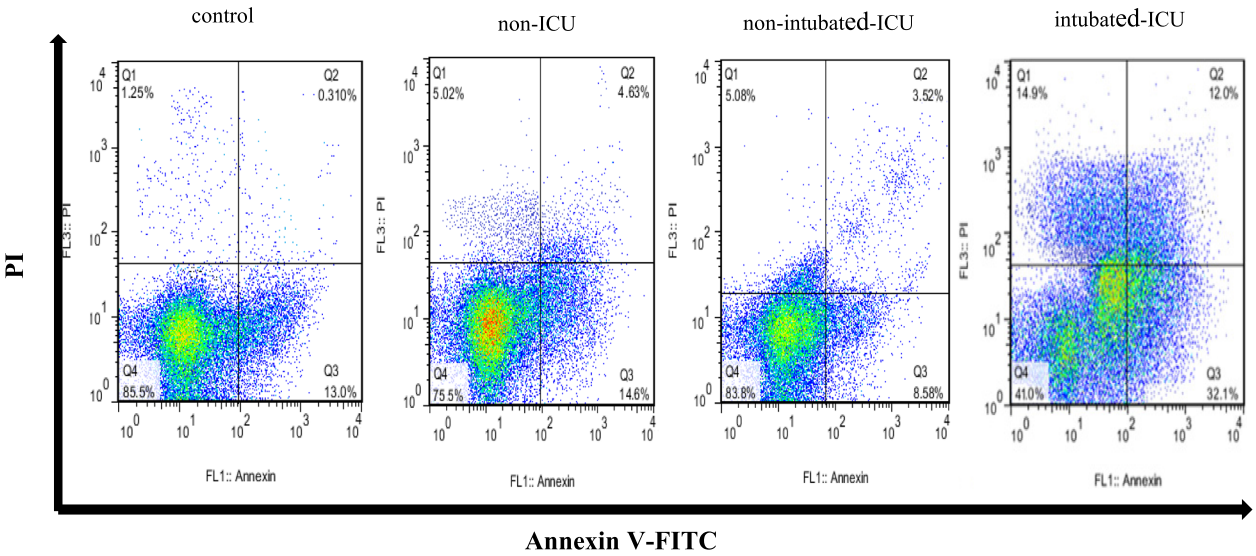

Fig. 2 Comparison of apoptosis in elderly patients with COVID-19 in 2020: significant difference in different flow cytometry levels of apoptosis in the 2 control groups and the intubated groups ($p < 0.01$). Analysis of apoptosis levels in peripheral blood mononuclear cells (PBMC) from controls and all elderly patients hospitalized with COVID-19 in non-intubated ICU and intubated ICU and non-ICU departments

there was a substantial decrease ($p < 0.01$) in the influenza, intubated-ICU, and non-intubated-ICU groups.

Discussion

The problem, which started in 2019 and continues to pose a threat to human society, is related to the respiratory system, which is prone to serious diseases. The cause of this issue is SARS-CoV-2, an unknown and new virus that caused the COVID-19 pandemic. The disease has a wide range of symptoms, notably difficulty breathing, a nonproductive cough, and a high fever. If we look for clues to find the cause of lymphopenia in serious diseases, we come across a natural process called apoptosis, and now we are faced with the dreaded disease called COVID-19. The process of apoptosis is still unknown to us in many diseases because there is no clear explanation for most of the stages of apoptosis. Nonproductive coughing, high fever, and shortness of breath are important symptoms of this disease. However, the virus can facilitate the apoptosis of persistent viral infection in host cells and spread the virus [32–34]. According to the findings, it is possible that the induction of lymphocyte apoptosis by inflammatory cytokines is directly related to the lymphopenic phase in deaths from COVID-19 [24, 34]. The association of lymphopenia with COVID-19 can no longer be ignored, and this issue is similar to the findings of a study in patients with H5N1, where lymphopenia is caused by T cell apoptosis [35]. However, there have been no significant studies on lymphopenia caused by COVID-19 [36]. We have attempted to study the relationship between lymphopenia caused by COVID-19 and lymphocyte apoptosis. Most of the hospitalized COVID-19 patients had lymphopenia, and according to the results, these patients had a decrease in lymphocytes. The occurrence and progression of severe COVID-19 depend on how the immune system responds to the virus in infected patients. The apoptosis pattern was measured by separating the circulating blood lymphocytes of the control group and COVID-19 patients by density gradient centrifugation. Flow cytometry can measure the level of apoptosis using the Annexin V-FITC/PI detection method. For the appropriate gating and background staining, cells were stained separately for each sample. The results show that COVID-19 normally strongly affects T cells, and lymphocytes cause various immune system disorders [37]. Inflammatory mediators can damage the immune system and, indirectly, cause lymphopenia. The onset of lymphopenia can be due to COVID-19 as well as the age of the patient and other conditions such as infection. Among several findings, it was concluded that patients with moderate cases of COVID-19 had more lymphocytes than patients with severe cases of COVID-19 [9, 11, 22]. Viral infections trigger the production of antibodies, can inhibit the development of hematopoietic cells, and ultimately reduce the production of things like T cell differentiation, all processes that explain the programmed death process in COVID-19. The results highlight that the involvement of intrinsic and extrinsic apoptosis pathways is something that is effectively upregulated by COVID-19, and that's what MERS-CoV did [38]. One of the reasons why body cells undergo apoptosis when exposed to the influenza virus is that

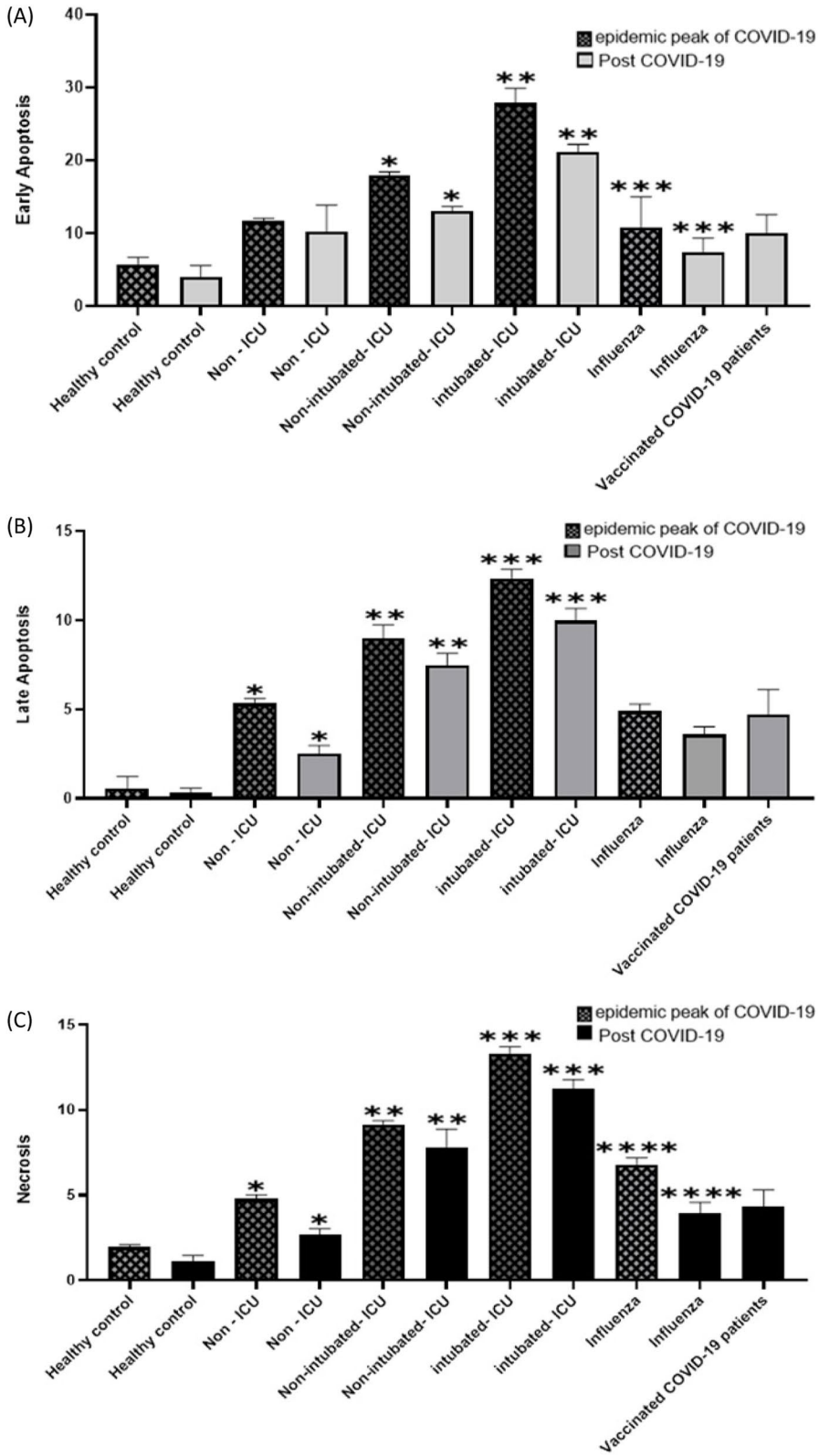

Fig. 3 Col: one-way ANOVA. The mean apoptosis cell percentage obtained from all elderly patients with COVID-19 and elderly controls in 2020 and 2022 is shown. This comparison was done in 3 stages of the flow cytometry technique, including **A** early apoptosis, **B** late apoptosis, and **C** necrosis, and showed that the apoptosis cell percentage increases with the progress of the disease. Differences between mild and severe cases are by * indicating significance ($p < 0.01$)

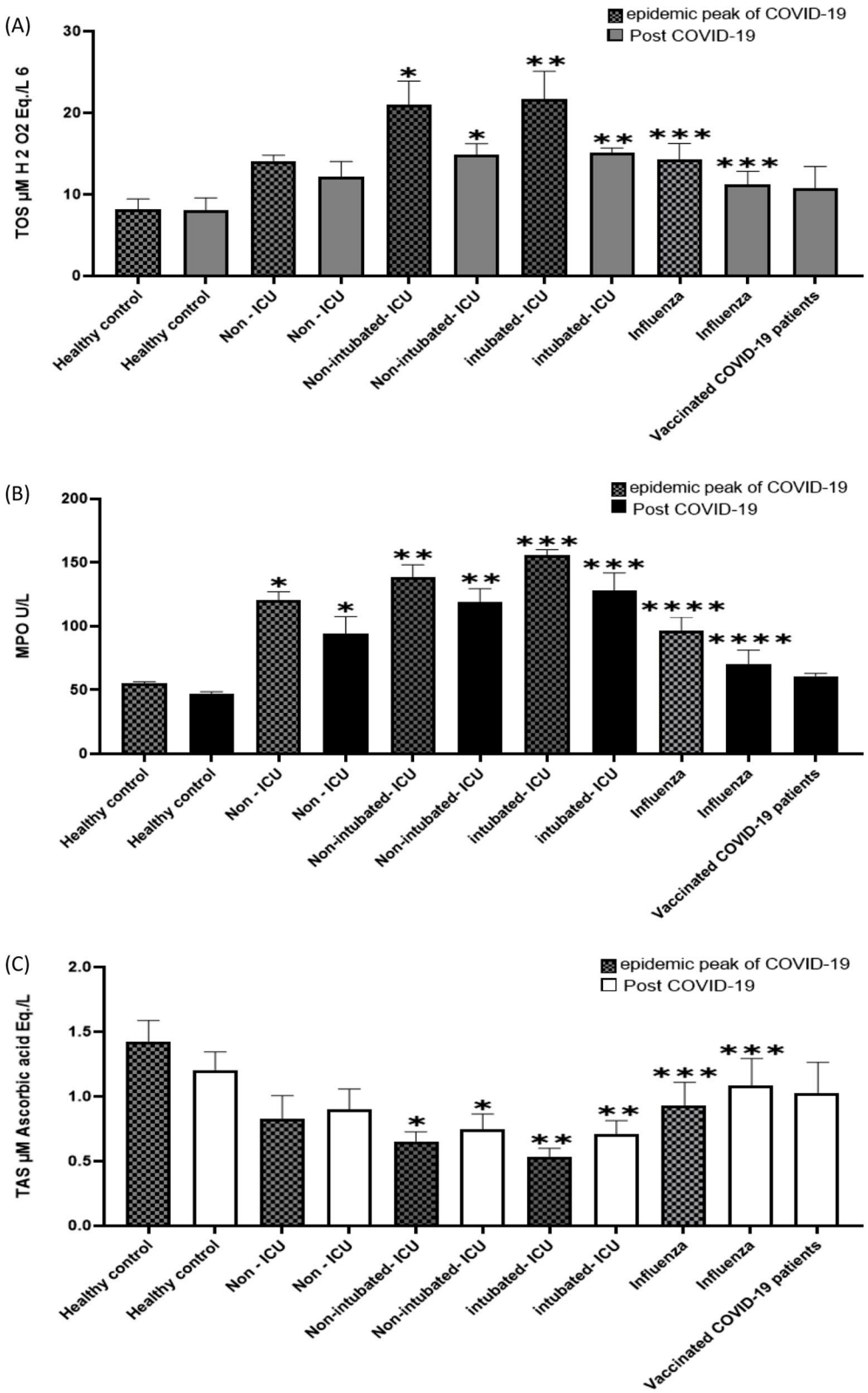

Fig. 4 Col: one-way ANOVA. The average percentage of oxidative damage found in all aged COVID-19 patients, elderly controls, and influenza patients in 2020 and 2022 is displayed. Three oxidative stress biomarkers were compared: **A** total oxidant status (TOS), **B** myeloperoxidase (MPO), and **C** total antioxidant status (TAS). Significant differences ($p < 0.01$) between mild and severe cases are indicated by an asterisk (*)

CD8 + CTLs directly attack and lyse infected cells, or it leads to an increase in the expression of death receptor ligands, and all these events are to clear the influenza virus. Infiltrating apoptosis leukocytes have been observed in the lungs of patients infected with lethal human influenza (IAV) and highly pathogenic influenza (HPAI) viruses such as the H5N1 virus. In previous studies, patients with a virulent IAV influenza infection had cellular damage and apoptosis, and this result was well demonstrated in a lung autopsy. What exactly is the nature of lymphocyte apoptosis? Is questionable and needs to be investigated. Is this apoptosis a part of the viral infection pathway, or is it a part of the immune response of the host infected with the virus? In another previous study, it was shown that influenza can lead to DNA fragmentation and activate the induction of apoptosis. One of the reasons that the induction of apoptosis is activated is the expression of cytokines such as FasL, TNF, and TNF-related apoptosis-inducing ligand (TRAIL), which attract apoptosis receptors in IAV-infected cells. The lungs of patients infected with influenza viruses, especially the dangerous type of human IAV, have infiltrating apoptosis leukocytes. The consequences of cell death caused by influenza IAV infection depend on the type of cells affected. Are respiratory epithelial cells damaged, or are monocytes, macrophages, or lymphocytes? In order for the influenza virus IAV to spread easily in cell lines such as MDCK, Vero, and human epithelial cell line A549, the activation of caspase 3 is important [24]. The avian influenza virus (H5N1), which can be transmitted to humans through the NS1 protein, leads to the induction of apoptosis and damages human airway epithelial cells. This pathway depends on the activation of caspase 3. Lethal H5N1 influenza virus infection induces apoptosis in IAV-specific CD8 + T cells in draining lymph nodes in the lung. This process has negative and destructive effects on the body's antiviral defense. H5N1 IAV induces a destructive immune response against apoptosis, and this happens through the prolongation of virus replication in human alveoli and primary bronchial epithelial cells. Most of the RNA viruses lead to infection in cells. They do this through anti-apoptosis and apoptosis-inducing processes to facilitate virus propagation. On the other hand, the infected host cell also tries to use apoptosis as part of the antiviral response to destroy the generation of infectious viruses. It has not yet been precisely determined whether the process of apoptosis can be beneficial or harmful to the host, and this requires further research. In a simultaneous study, autologous human PBMC were exposed to respiratory syncytial virus (RSV) and influenza IAV, and apoptosis was induced in both, but apoptosis was much less in RSV. The role of monocytes and macrophages in inducing lymphocyte apoptosis is important for all types of viruses. Several studies have been cited as examples, including bovine viral diarrhea that resulted in the induction of lymphocyte apoptosis by infected macrophages. Macrophages infected with the African swine fever virus also induce lymphocyte apoptosis [24, 39–42]. After the influenza virus leads to infection, neuraminidase is expressed in the infected cells of the host. Macrophages infected with RNA viruses such as the caprine arthritic encephalitis virus (CAEV) lead to the activation of the apoptosis process in the infected individual's lymphocytes. For a long time, it was believed that monocytes and macrophages play a very important role in the antiviral defense of an infected person, but today it has been found that these lead to the production of antiviral factors, such as interferon. Monocytes and macrophages in the respiratory tract and lungs of people infected with the IAV influenza virus directly communicate with the infected epithelial cells and finally lead to the induction of apoptosis in the epithelial cells. Epithelial cells, respiratory cells, and alveoli are especially attacked by infectious viruses such as influenza IAV, and this virus multiplies in these environments until apoptosis occurs [24, 43, 44]. In particular, studies have found that human macrophages and monocytes are highly sensitive to IAV infection [24]. The infectivity of IAV influenza viruses and their families is a constant threat to humans. It is very important to identify the similarities between this influenza virus and COVID-19 and their pathogenesis. People with COVID-19 who received the vaccine experienced a decrease in apoptosis in the wave after COVID-19. There are many unknowns about the process of pathogenesis and apoptosis of lymphocyte cells caused by dangerous infectious viruses such as influenza and COVID-19. But this result cannot be ignored: the death of lymphocyte cells due to apoptosis is actually a final defense by the host against these dangerous infectious viruses and the host's survival effort. In a work by Singh et al. [45], it was demonstrated that mitochondrial disruption in SARS-CoV-2-infected lung cell lines promoted inflammation and severity in COVID-19-related sepsis. The consequences of COVID-19 include a marked increase in tissue inflammation, oxidative damage, and ultimately DNA damage [46, 47]. The findings of these investigations are consistent with our finding of significant DNA damage in older COVID-19 patients and senior influenza patients. Oxidative stress is caused by disturbances in the steady state of ROS formation and removal. Oxidizing DNA, membrane lipids, and structural proteins are characteristic of it, as they undermine and make it more difficult for cells to heal themselves [48]. Elevated ROS levels caused by respiratory virus infections are linked to cytokine production, oxidative stress or redox imbalance, and cellular damage. Virus

infections generate large amounts of free radicals (ROS), and the virus must spread in order for these antioxidant systems to deplete the ROS [49, 50]. Recent studies have shown that when oxidative stress is present, people with lung dysfunction and the cytokine storm from SARS-CoV-2 infection have a higher severity of COVID-19 [51, 52]. The results of our investigation show that, whereas TAS levels were lower in COVID-19 patients, TOS levels were significantly higher. These conclusions were confirmed by the literature. Neutrophils that have been triggered generate a pro-inflammatory enzyme called MPO, which is an important enzyme. One feature of COVID-19 is that it induces MPO production from encroaching neutrophils, which triggers several pathways that control cytokines and produce reactive oxygen species [53]. According to studies by Guéant et al. [54], elevated MPO-DNA blood levels were discovered in positive individuals, indicating that this is a sensitive marker of the early stage of COVID-19. According to our research, COVID-19 had greater MPO levels. These results are consistent with the literature. This study looked at DNA damage, oxidative stress, and peripheral blood lymphocyte apoptosis in COVID-19 patients. The post-COVID-19 influenza group showed a decrease in DNA damage, apoptosis, TOS, and MPO levels in all laboratory tests examined, with the exception of TAS. This may suggest that psychological stress during severe assaults is the cause of oxidative damage. Other infectious diseases, like influenza, have seen an upsurge in DNA damage and cell apoptosis as a result of the COVID-19 pandemic. Peripheral blood lymphocyte apoptosis levels in all three groups of COVID-19 vaccination recipients, influenza patients, and non-ICU patients were nearly identical and did not differ substantially from one another in the post-COVID-19 period ($p > 0.01$). Cell apoptosis can be brought on by oxidative stress. ($p < 0.01$), induced oxidative stress, DNA damage, and cell death in COVID-19 patients can predict prognosis and direct treatment approaches. According to our findings, the SARS-CoV-2 virus is an undiscovered pathogen that can cause lymphocytes to die. Elderly and deceased individuals had higher percentages of underlying conditions, particularly cardiovascular disorders. COVID-19, with two or more comorbid conditions, posed a greater danger. Clinical indicators and manifestations are as follows: dyspnea and anorexia were more common in the deceased. Our study discovered that while antioxidant defenses wane, oxidative stress and inflammation are higher in COVID-19 patients. Thus, prognostic and therapeutic approaches in COVID-19 patients can be guided by factors such as DNA damage, inflammation, and oxidative stress. These findings have important implications for the crucial necessity of customized therapies and monitoring plans for older adults following COVID-19. Patient care and outcomes can be greatly improved by comprehending how SARS-CoV-2 affects cell health and by using apoptosis levels as a prognostic indicator. By incorporating these biomarkers into clinical practice, healthcare providers can better assess the severity of the disease, optimize treatment approaches, and improve overall patient management. Moreover, the research sheds light on the multifaceted nature of the SARS-CoV-2 infection and its implications for public health. By recognizing the significance of apoptosis levels in peripheral blood lymphocytes, healthcare systems can refine risk stratification strategies and allocate resources more effectively. These insights not only contribute to individualized care but also inform broader public health initiatives aimed at mitigating the impact of COVID-19 on vulnerable populations. The study's conclusion emphasizes the value of further investigation and creativity in overcoming the difficulties caused by SARS-CoV-2, especially when it comes to older people. Through the use of biomarkers, such as peripheral blood lymphocyte apoptosis levels, healthcare providers can improve patient outcomes, optimize treatment regimens, and evaluate the effectiveness of immunizations and other preventive measures. These results highlight the vital role that proactive healthcare approaches and individualized therapy play in tackling the complexity of COVID-19 and enhancing public health outcomes in general. Suggested reading for health policymakers is as follows: especially for older populations, health planners should give top priority to integrating peripheral blood lymphocyte apoptosis levels as a standard diagnostic for determining post-COVID-19 prognosis. Policymakers can improve risk assessment, treatment planning, and other related processes by including this measure in healthcare guidelines and protocols. Additionally, it is recommended that policymakers endorse research endeavors that endeavor to clarify the significance of apoptosis levels in assessing the effectiveness of vaccines against SARS-CoV-2. Policymakers can improve public health responses to the ongoing epidemic, optimize vaccination methods, and educate on evidence-based decision-making by funding studies that examine the relationship between biomarker levels and vaccine efficacy. This proactive strategy will support healthcare systems and aid in the creation of focused treatments that are suited to the requirements of vulnerable groups, like the elderly.

Conclusions

The research results emphasize the potential for serious cell damage and the ongoing hazard that SARS-CoV-2 poses to the older population even after COVID-19 recovery. Promising indicators for determining patient

prognosis include measuring oxidative stress, DNA damage, and apoptosis levels in peripheral blood cells. These biomarkers provide information about the efficacy of therapies meant to lower death rates, in addition to helping to anticipate outcomes. Additionally, the research indicates that tracking apoptosis levels can be a useful method for assessing how well SARS-CoV-2 vaccines are working. Keeping an eye on oxidative stress can prevent additional DNA damage. DNA damage monitoring can prevent further apoptosis. Monitoring apoptosis can prevent further deaths. It can be said that all these biomarkers are related.

Acknowledgements
The financial support of this research was done by the student. This research was carried out in the form of an approved research plan at Damghan Islamic Azad University.

Authors' contributions
M.M and Ma.M conceived the original idea and designed the project. E.A participated in the design and executed the experiments. A.A and A.H. discussed the results and strategy. E.A wrote the manuscript. All authors reviewed, edited, and approved the final manuscript.

Funding
[This project did not receive any financial support from any non-profit, government, or for-profit financial institutions.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Before data collection, written and informed consent was obtained from all the patients participating in the research, and the patients entered the study with their personal consent. All procedures performed in the study involving human participants were by the ethical standards of the institutional and national research committee and with the 1975 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Biomedical Research Ethics Committee of Damghan Islamic Azad University, which issued the study's code of ethics (IR.IAU. DAMGHAN. REC.1400.005).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 22 May 2024 Accepted: 7 August 2024
Published online: 09 September 2024

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